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14. ABSTRACT

The major goal of this project is to determine if the rhodacyanine analog dye, MKT-077, can be used to inhibit breast cancer cell oxygen metabolism and raise tumor oxygen levels, thereby radiosensitizing the tumor. During the third year of the project, we completed the proposed in vitro experiments and much of the proposed in vivo work. We have shown that R3230Ac breast cancer cells rapidly take up MKT-077 in a dose-dependent manner, although cells raised on air take up more drug than cells grown under hypoxic conditions. Interestingly, low oxygen levels during drug exposure slow the uptake of MKT-077, regardless of the oxygen level at which the cells were raised. We have also shown that MKT-077 can inhibit cellular oxygen metabolism by up to 70% in R3230Ac cells grown on air (21% O2), 2.5% O2, or 1% O2 in a dose-dependent fashion. The magnitude of consumption inhibition was correlated with cellular drug uptake. Finally, and most importantly, we have demonstrated that IV infusion of 10 mg/kg MKT-077 over 60-80 minutes results in a significant increase in tumor oxygen tension (PO2) and a decrease in hypoxic fraction in R3230Ac mammary tumors growing in Fischer 344 rats. Since lower doses and the same dose infused over a much shorter time did not alter tumor PO2, dose scheduling is extremely important for MKT-077 efficacy. We have requested a no-cost extension to further explore the dosing in Task 3 and possibly perform the proposed radiation study (Task 4).

15. SUBJECT TERMS
Breast cancer, radiosensitizer, metabolic inhibition, MKT-077, tumor oxygenation

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INTRODUCTION

The driving hypothesis behind this project is that the rhodacyanine dye analog MKT-077 can be used to inhibit breast cancer cell respiration, leading to increased oxygen levels within the tumor and a resultant increase in tumor radiation response. This central hypothesis is built upon the following rationale. First, the response of tumors to radiation therapy is dependent upon oxygen levels,[1] and tumors with low oxygen levels (i.e., hypoxic tumors) are radioresistant.[2] Second, over 60% of human breast tumors are severely hypoxic.[3-5] Third, of the two methods to increase oxygen levels, inhibition of tumor cell oxygen consumption is theoretically a better method than increasing the oxygen supply.[6, 7] Fourth, MKT-077 has been shown to inhibit mitochondrial respiration in some cancer cell types.[8] Therefore, we expect to be able to decrease oxygen consumption and increase the oxygen levels in breast carcinoma by infusing MKT-077. If oxygen can be increased, then infusion of MKT-077 before radiotherapy should increase radiation response and permit lower levels of radiation to be used for preoperative treatment of locally advanced breast cancer (LABC). Lower radiation doses would result in less collateral damage to normal tissue and better cosmetic outcome. In addition, it might be possible to shorten treatment schedules, which would be a benefit to both patients and physicians. Finally, the use of the radiosensitizer could result in better locoregional control in LABC patients. This could increase the number of these women eligible for breast conserving treatment and lead to their improved survival. The use of MKT-077 in combination with radiotherapy should also be useful in patients with early stage breast cancer, who are receiving radiation as part of breast conserving treatment[9, 10] or locoregional post-mastectomy radiotherapy.[9, 11]

BODY

As noted in the Annual Report for Year 2, we have been concurrently performing Tasks 1, 2, and 3 in our Statement of Work, which we have had to slightly modify. The changes involved were reported in the first two Annual Reports, and a request to alter the Statement of Work was recently submitted. First, since we encountered some difficulties in Year 1 (see Year 1 Report), we had to alter the methods used in Tasks 1 and 2. After beginning Task 3, we were having difficulty growing tumors in the nude rat model, and we had to switch to the R3230Ac rat breast tumor growing in Fischer 344 rats (see Year 2 Report). Because we are now using rat R3230Ac cells in our *in vivo* experiments, we decided to use the R3230Ac cell line for the *in vitro* work in Tasks 1 and 2 as well. Over the past year, the *in vitro* work in Tasks 1 and 2 have continued to be highly successful, and we have essentially completed those experiments. MKT-077 is clearly taken up by the R3230Ac cells (Task 1) and impressively inhibits their oxygen consumption *in vitro* (Task 2), although both parameters are affected by the oxygen level at which the cells were grown. We have also been performing experiments addressing Task 3 in the R3230Ac model. Initially we had difficulty showing any consistent effect of the drug on tumor oxygen tension (PO_2), but over the past several months we have clearly demonstrated that the drug results in significant improvement of tumor oxygenation if enough of it reaches the tumor. Overall, we have proven the three hypotheses in Tasks 1 through 3. These new data will be presented at the 2008 Era of Hope Meeting in June 2008 by Mr. John Chunta, the graduate student who has performed most of the experiments in this project. We have requested a no-cost extension to optimize the dosing in Task 3, as well as to perform Task 4 with an optimized protocol. Details of our progress on each specific task are given below.

Revised Task 1. To determine the transport parameters and cellular uptake of MKT-077 in breast cancer cells at different drug concentrations and oxygen levels (Months 1-18):

Model: We will use *in vitro* suspensions of rat R3230Ac mammary adenocarcinoma cells, a rat breast cancer cell line.

Methods: Cell suspensions will be placed in a specially built, water-jacketed, plexiglas chamber and exposed to media bubbled with different amounts of oxygen. We will add different concentrations of MKT-077 to the chamber and take samples of the cell suspension at different times. The level of MKT-077 in the samples will be determined spectrophotometrically, and the amount of MKT-077 taken up by the cells is expressed as ng MKT-077/100,000 cells.

Objective:

1) Determine the effect of oxygen level on cellular uptake rate of MKT-077 as a function of drug dose in breast cancer cells.

Progress on Task 1: Using the methods outlined above, we have determined the uptake rate of MKT-077 in cell suspensions of R3230Ac mammary adenocarcinoma cells at different doses and at different oxygen levels. Some of these data were presented in very preliminary form in the last report, but the experiments have now been completed.

Results from the experiments, in which cells were grown on air and were then exposed to 2, 4, or 6 μg/ml MKT-077 at time zero in air-equilibrated medium, are shown in Figure 1. The cells take up the drug relatively quickly, and then the uptake rate begins to level off. The fast uptake rate is important, since the concentration of drug exposed to the tumor will diminish after infusion into the rat's circulation.[12] The uptake of MKT-077 is clearly dose-dependent and time-dependent. We are working on modeling the uptake for inclusion in an upcoming manuscript. The individual curves can be fitted relatively well by a simple monoexponential, but are also adequately described by a Michaelis-Menten equation. Regardless of the dose used, near maximal uptake is achieved after 60-90 minutes of drug exposure.

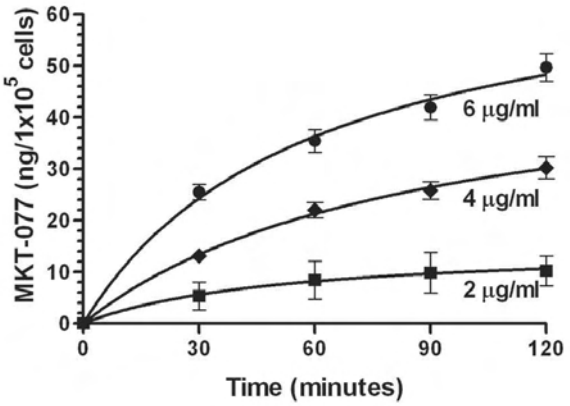

Figure 1. [MKT-077] of R3230Ac cells in suspension after addition of 2, 4, or 6 μg/ml of MKT-077 to the medium. Cells were grown on air and were exposed to air during the experiment. Mean ± SEM. N=6-7.

Since *in vivo* tumors grow at much lower oxygen levels and drug uptake would occur in the presence of much less oxygen, we next attempted to determine the impact of the oxygen level during growth and during drug exposure on R3230Ac MKT-077 uptake. When cells were grown under hypoxic conditions (1% or 2.5% O_2) and exposed to MKT-077 in air-saturated medium, drug uptake significantly increased as a function of the oxygen level at which the cells were grown (Figure 2). Since increased tumor MKT-077 uptake is thought to be at least partially due to a higher mitochondrial membrane potential in malignant cells compared to normal cells,[8] this decreased ability of cells grown at lower oxygen levels to take up the drug may be related to differences in the mitochondria between cells grown at different oxygen levels. Interestingly, there was no difference in mitochondrial content among the cells grown at 1%, 2.5%, or 21% O_2 (data not shown), indicating the uptake difference is not caused by a difference in mitochondria number. Therefore, the uptake may be related to a difference in mitochondrial activity or it is related to a factor other than differences in the mitochondria. When cells were grown under hypoxic conditions (1% or 2.5% O_2) and

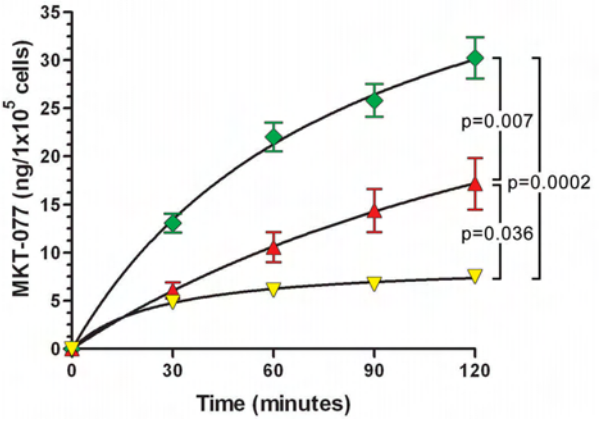

Figure 2. Uptake rate of MKT-077 by R3230Ac cells grown on air (◆), 2.5% O_2 (▲), or 1.0% O_2 (▽) and exposed to 4 μg/ml MKT-077 in air-saturated medium. Mean ± SEM, N=6-7.

exposed to MKT-077 in medium equilibrated to gas at the same oxygen level or to gas equilibrated with air, drug uptake was again dependent on oxygen level (Figure 3). Regardless of the oxygen level at which the cells were grown, the presence of more oxygen during drug exposure resulted in an increase in cellular uptake. These findings are again consistent with the idea that mitochondrial properties are altered by ambient oxygen levels, since MKT-077 uptake is thought to be mitochondria-dependent.[8] The implications of these results for our *in vivo* studies are significant, since they indicate that the more hypoxic tumor cells will take up the drug more slowly. While this is not an ideal situation, the *in vivo* success of MKT-077 really depends on the ability of normoxic or slightly hypoxic cells to take up the drug. If these cells take up the drug and their oxygen consumption decreases, then the diffusion distance of oxygen should increase and the hypoxic cells at a distance from the vessels should be better oxygenated. The data also indicate that it might be beneficial to use another means, e.g., breathing of hyperoxic gases, to try to increase tumor PO_2 levels during the infusion of MKT-077.

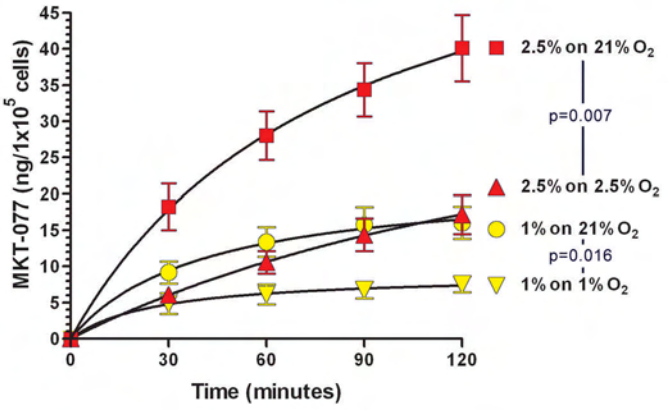

Figure 3. Uptake rate of MKT-077 by R3230Ac cells grown on 2.5% O_2 (red symbols) or 1.0% O_2 (yellow symbols) and exposed to 4 µg/ml MKT-077 in medium saturated with air (21% O_2) or the gas at which they were grown, e.g., "1% on 1% O_2": cells grown on 1% O_2 and exposed to drug at 1% O_2. Mean ± SEM, N=6-7.

The results of these experiments show that MKT-077 is rapidly taken up by R3230Ac breast cancer cells. In answer to Objective 1, we have shown that the uptake is dose-dependent and that hypoxic cells take up less drug than cells that are better oxygenated. These results will be included in a manuscript on the *in vitro* effects of MKT-077 that is being written by Mr. John Chunta, the graduate student who has performed these experiments. The paper has been delayed until now, since we only recently completed the oxygen consumption experiments in Task 2.

Revised Task 2. To determine the effect of MKT-077 on tumor metabolism in breast cancer cells at different drug concentrations and oxygen levels (Months 6-24):

Model: We will use *in vitro* suspensions of rat R3230Ac mammary adenocarcinoma cells, a rat breast cancer cell line.

Methods: Suspensions of cells grown at different oxygen levels will be placed in a specially built, water-jacketed, plexiglas chamber. A Clark-type oxygen electrode will be positioned in the suspension within the inner chamber. The chamber will be sealed, and oxygen tension (PO_2) will be recorded continuously. After at least 20 minutes, concentrated MKT-077 will be added to the inner chamber to bring the drug concentration to the desired level. The recording will be continued for another two hours or until the PO_2 drops below 20 mm Hg. Oxygen consumption will be calculated from the PO_2 recording.

Objectives:

1) Determine if exposure of breast cancer cells to different doses of MKT-077 causes a decrease in oxygen consumption. We predict that higher doses of MKT-077 will cause a greater decrease in oxygen consumption.
2) Determine if the MKT-077-induced decrease in oxygen consumption is a function of the oxygen level at which the cells were grown.

Progress on Task 2: Using the methods outlined above, we have determined the effect of MKT-077 on cellular oxygen consumption in cell suspensions of R3230Ac mammary adenocarcinoma cells at different doses and at different oxygen levels.

The R3230Ac cells were grown to 90% confluency in air in a standard incubator or in 1% O_2 or 2.5% O_2 in a hypoxia chamber. The cells were then trypsinized, counted, and resuspended in fresh tissue culture media to a concentration of $1x10^6$ cells/ml. Five and one-quarter ml of the suspension were placed in the inner chamber of a specialized tonometer that is heated to 37°C by circulating warm water. A Clark-type oxygen electrode (InO2 Dissolved Oxygen System, Innovative Instruments, Inc., Tampa, FL) was positioned in the suspension within the inner chamber. The suspension was exposed to air and allowed to equilibrate by gently stirring the suspension with a small magnetic stir bar. We could not perform these experiments at lower oxygen levels, because medium equilibrated at lower oxygen concentrations dropped to lower levels too quickly, and the oxygen tension (PO_2) in the medium could not be tracked long enough to reliably determine oxygen consumption. The chamber was then sealed, and oxygen tension (PO_2) was recorded continuously. After at least 20 minutes, concentrated MKT-077 was added to the inner chamber to bring the drug concentration to the desired level. The recording was continued for another two hours or until the PO_2 dropped below 20 mm Hg. In preliminary experiments, we determined that consumption sometimes changed when the PO_2 dropped this low in undisturbed cells. Examples of experiments performed at different MKT-077 doses on cells grown on 2.5% O_2 are shown in Figure 4. Addition of saline (0 µg/ml), did not change the slope of the line describing PO_2 as a function of time, indicating no change in O_2 consumption. Addition of MKT-077 clearly changed the course of the PO_2 decrease, and showed that the O_2 consumption rate was decreased by the drug. Qualitatively, the higher the dose of MKT-077, the shallower the slope of the curve appeared, i.e., the larger the decrease in consumption became (Figure 4).

As noted in previous reports, we have developed a mathematical model to quantify these changes in PO_2. We first assume that the oxygen consumption rate, q [ml O_2/(10^5 cells min)], changes monoexponentially from q_1 to q_2, following addition of MKT-077 at time 0:

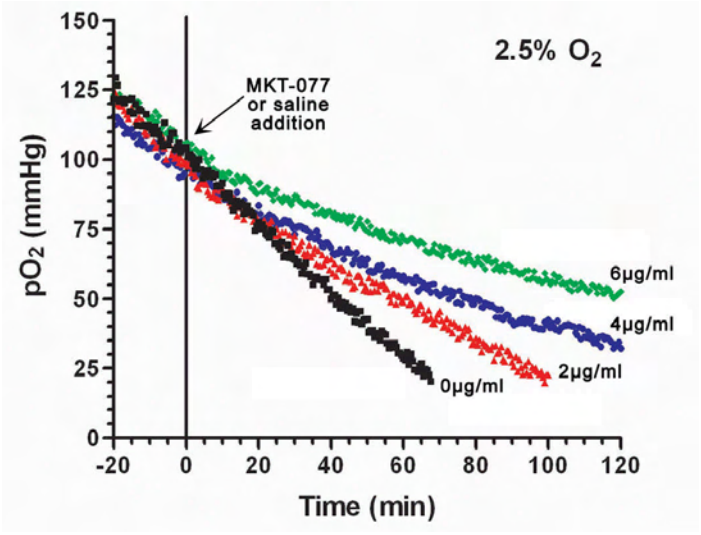

Figure 4. PO_2 measured in R3230Ac cell suspension before and after addition of different concentrations of MKT-077 (0, 2, 4, or 6 µg/ml) to the media at t=0 minutes. R3230Ac cells grown on 2.5% O_2.

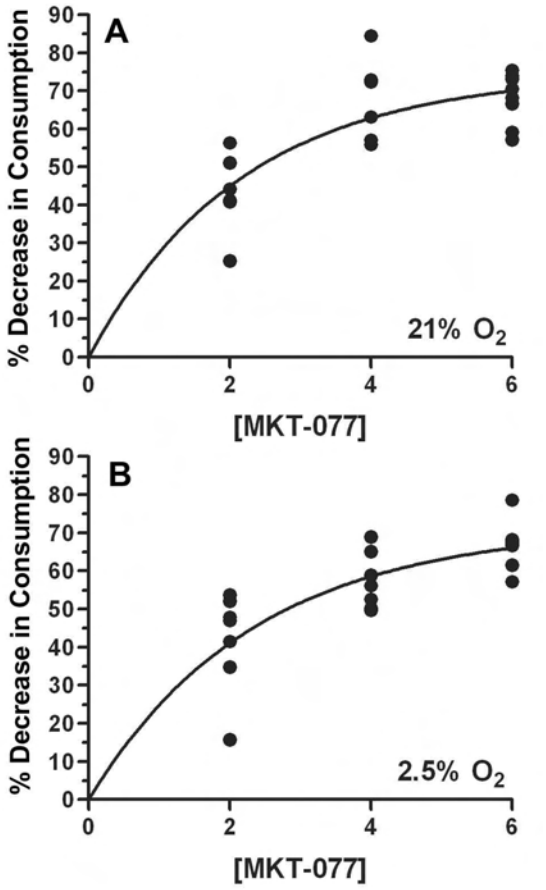

Figure 5. Decrease in O_2 consumption determined from the model after R3230Ac cells grown on air (A) or 2.5% O_2 (B) are exposed to different concentrations of MKT-077. Each point represents an individual experiment.

$$q(t) = q_1, -20 \le t < 0$$
$$q(t) = q_2 - (q_2 - q_1)e^{-t/tc}, t \ge 0$$

where q_1 = basal O_2 consumption rate [ml O_2/(10^5 cells min)], q_2 = O_2 consumption rate at steady state after MKT-077 addition [ml O_2/(10^5 cells min)], and tc = time constant of change in q (min). This function can be substituted into the mass balance equation for this system: k(dP/dt) = -Cq(t), where k is the oxygen solubility of the suspension (ml O_2/ml suspension/mm Hg), P is the PO_2 (mm Hg), t is time (min), C is the concentration of cells ($1x10^6$ cells/ml suspension), and q(t) is the oxygen consumption rate as a function of time given by the above equations. By integrating this function, PO_2 can be expressed as a function of time and four unknown parameters (P_0, q_1, q_2, and tc):

$$P(t) = P_0 - \left(\frac{C}{k}\right)q_1(t + 20), -20 \le t < 0$$
$$P(t) = P_0 - \left(\frac{C}{k}\right)(20q_1 + q_2 t) + \left(\frac{C}{k}\right)(q_2 - q_1)tc\left(1 - e^{-\left(\frac{t}{tc}\right)}\right), t \ge 0$$

where P_0 = initial PO_2 at time t = -20 min (mm Hg). Using MatLab software (MathWorks, Inc., Natick, MA), we have written a program to fit the experimental PO_2 data to this model. Examples of fits of the model to experimental data were shown in the Year 2 report, and the model clearly fitted the data well. We have continued to apply it to our experimental data in Task 2. From these model fits, the change in oxygen consumption could be estimated at any given time. In subsequent figures, the oxygen consumption decreases reported are the steady-state changes, i.e., after 1-2 hours of drug exposure.

Over the past year, we have completed the experiments originally planned to address Task 2. When cells were grown on air and then exposed to 2, 4, or 6 μg/ml MKT-077, the oxygen consumption of the cells decreased by as much as 60-70% in a dose-dependent fashion (Figure 5A). As noted in last year's report, the time course of the change in oxygen consumption also appears to be time dependent, with higher MKT-077 doses resulting in faster changes (see Year 2 report). Similar results, in terms of relative MKT-077-induced changes in oxygen consumption, were found for cells grown at 2.5% O_2 (Figure 5B). Indeed, when the changes in oxygen consumption induced by exposure to 4 μg/ml MKT-077 were compared for cells grown at 1%, 2.5%, or 21% O_2, there was no difference in the relative decrease in consumption (Figure 6A). However, we found that cells grown on 1%, 2.5%, or 21% O_2 had different levels of basal oxygen consumption. As might be

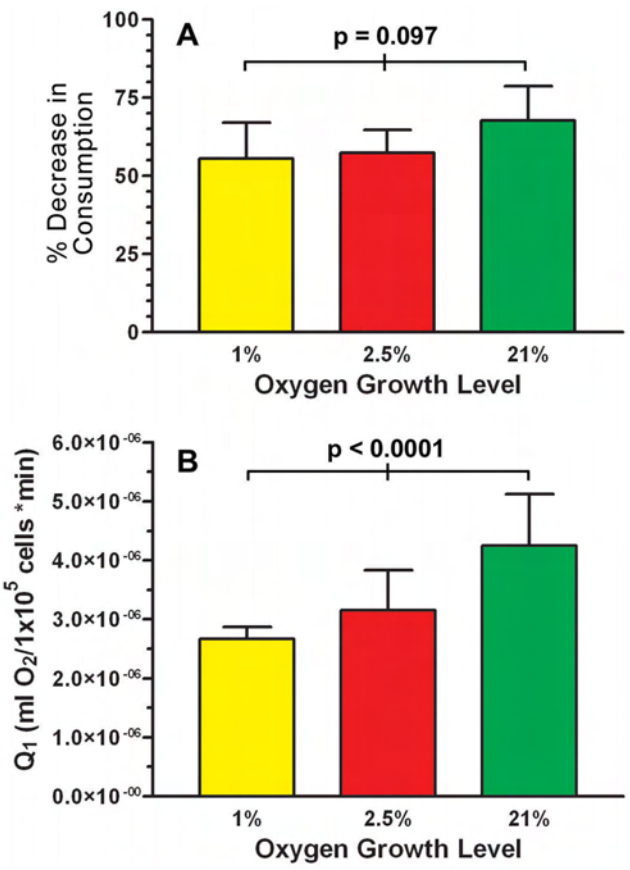

Figure 6. A. Percent decrease in O_2 consumption of R3230Ac cells after exposure to 4 μg/ml MKT-077. Mean ± SEM, N=6-7. B. Basal O_2 consumption rate (q_1 in model) for R3230Ac cells grown on 1% O_2, 2.5% O_2, or air (21% O_2). p values calculated using an ANOVA with Bonferroni's multiple comparison test.

predicted, cells grown on 21% O_2 had a significantly higher level of basal consumption compared to cells grown at the lower oxygen levels (Figure 6B). There was no difference between cells grown at 1% and 2.5% O_2 (Figure 6B). Since we found no difference in mitochondrial protein level in these cells (data not shown), this difference in basal consumption is most likely due to a difference in mitochondrial oxidative capacity, rather than a difference in mitochondria number. Taken together with the data in Figure 6A, it is clear that MKT-077 induces a larger change in absolute consumption in cells grown on air, although the relative change remains essentially constant, regardless of the growth conditions.

If MKT-077 is indeed altering the cellular oxygen consumption, we hypothesized that there should be a correlation between the cellular uptake of the drug and the MKT-077-induced change in consumption. To test this, we plotted the percent decrease in consumption predicted by our mathematical model at 30, 60, 90, and 120 minutes against the cellular uptake at those same time points (see Task 1). We could only do this for cells grown on 1% and 2.5% O_2, since cells grown on air consumed oxygen at such a high rate that we could not always gather consumption data for 90 or 120 minutes. There was indeed a strong correlation between MKT-077 uptake and the change in oxygen consumption (Figure 7). This is further evidence that uptake of the drug is causing the consumption change. In addition, it shows that the magnitude of the consumption change is directly proportional to the amount of MKT-077 in the cells. Interestingly, however, it takes less drug to bring about a given consumption decrease in cells grown at 1% O_2 than in cells grown at 2.5% O_2. This means that the oxygen consumption rate of the more hypoxic cells is more sensitive to MKT-077.

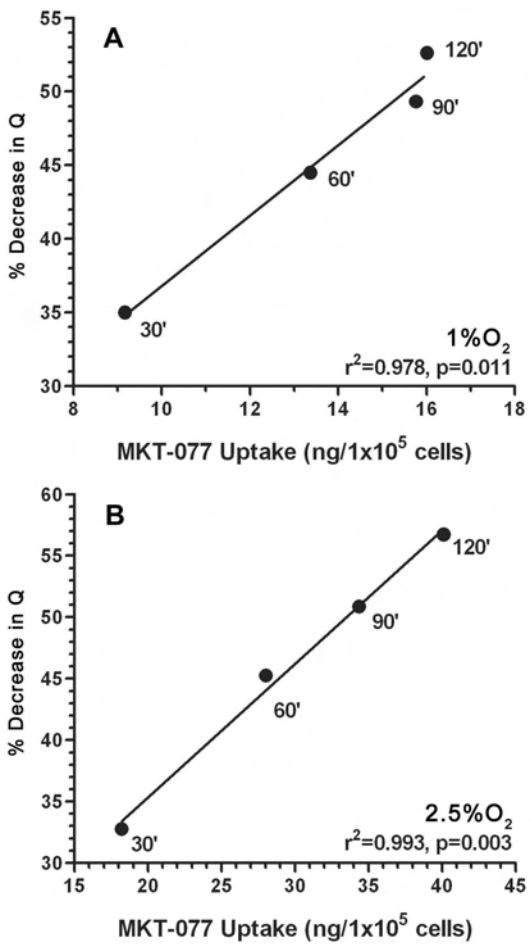

Figure 7. Correlation between the decrease in O_2 consumption determined from the model and the cellular uptake at different time points after addition of MKT-077. R3230Ac cells grown on 1.0% O_2 (A) or 2.5% O_2 (B) were exposed to 4 µg/ml MKT-077.

In summary, these data show that MKT-077 can inhibit oxygen consumption in the R3230Ac cells very effectively. They suggest that MKT-077 would be an extremely effective inhibitor of oxygen consumption *in vivo*, if enough drug can be delivered to the tumor. These are the experiments addressed in Task 3. The data gathered in Task 2 will be included in the manuscript on the *in vitro* effects of MKT-077 that is being written by the graduate student who has worked on this project, Mr. John Chunta.

Task 3. To determine if MKT-077 infusion can increase tumor PO_2 in orthotopic mammary tumors grown from rat R3230Ac breast cancer cells without altering tumor blood flow (Months 24-42):

Model: We will grow R3230Ac breast carcinomas orthotopically in the mammary fat pads of Fischer 344 rats. We estimate requiring 120 rats for this study.

Methods: We will use oxygen microelectrodes to measure PO_2 at a single site in the tumor following infusion of different doses of MKT-077. Tumor blood flow will be measured simultaneously at multiple sites using laser Doppler flowmetry to see if MKT-077 affects tumor perfusion. In a second set of experiments, we will measure PO_2 histograms in the tumors either 2 or 24 hours

after infusion of MKT-077 and determine the median PO_2 and other characteristics of the overall distribution.

Objectives:

1) Determine if MKT-077 infusion will transiently increase PO_2 in orthotopic breast tumors without altering local blood flow in a dose-dependent manner (Months 24-33). These are important measurements, since changes in perfusion can greatly affect tumor PO_2.
2) Determine if MKT-077 infusion will increase the median PO_2 in orthotopic breast tumors in a dose- and time-dependent manner (Months 34-42).

Progress on Task 3: After considerable difficulty finding a satisfactory animal model during the first 18-20 months of the grant (see Annual Reports 1 and 2), we are now successfully using the orthotopic R3230Ac rat mammary adenocarcinoma growing in the mammary gland of the Fischer 344 rat. This model has allowed us to complete most of the stated objectives in Task 3 during the past year.

For these *in vivo* studies, we have infused two doses of MKT-077 (7.5 mg/kg and 10 mg/kg) intravenously at a fast [1.25mg/(kg min) or 0.5 ml/(kg min)] and a slow [0.125mg/(kg min) or 0.05 ml/(kg min)] infusion rate. The fast infusion takes 6-8 minutes, while the slow infusion takes 60-80 minutes. In the Year 2 report, we showed preliminary data for the change in tumor PO_2 and blood flow following the fast infusion of 7.5 mg/kg MKT-077. Since that time we have completed most of the proposed experiments in Task 3.

To address Objective 1, we used oxygen microelectrodes to measure PO_2 at a single site in the tumor following infusion of the different doses of MKT-077 at the fast and slow rates. Tumor blood flow was measured simultaneously at multiple sites using laser Doppler flowmetry to see if MKT-077 affects tumor perfusion. The results are summarized for three of the dosing schedules in Figures 8, 9, and 10. In these experiments, baseline measurements were made from –20 to 0 minutes. At 0 minutes, the infusion was started and lasted until the total amount had been delivered. In each figure, the infusion period is shown between the vertical lines. It should be noted that the blood pressure data is pooled from all the experiments performed using the given schedule, including the histogram experiments performed later (see below). Control infusions of saline at the fast rate had no significant effect on tumor blood flow or tumor PO_2, although the blood pressure tended to rise significantly by the end of two hours after the infusion (data not shown). The fast infusion of 7.5 mg/kg showed no significant changes in blood pressure, tumor blood flow, or tumor PO_2 (Figure 8). Although blood flow did not change significantly, it did tend to increase or decrease after the infusion, as indicated by the larger standard deviations following the end of the infusion. The fast infusion of 10 mg/kg showed significant decreases in blood pressure of about 10 mm Hg at various times after the infusion (Figure 9). Again, tumor blood flow and tumor PO_2 remained unchanged, although blood flow again tended to be less stable after the infusion (Figure 9). Similar to the fast infusion schedule, the slow infusion of 7.5 mg/kg showed no significant changes in blood pressure, tumor blood flow, or tumor PO_2 (Figure 10). There was a tendency for blood pressure to drop slightly in these rats, but it was only significant at certain time points. Although the PO_2 did not change significantly, we did see a PO_2 increase in a subset of the rats, and the means were at least above zero for the first time. Given the variability in MKT-077-induced changes in tumor PO_2 at a single point, we decided we needed to complete the histogram experiments proposed in Objective 2, in order to determine if any of the dosing schedules result in a change in the PO_2 distribution across the entire tumor. Note: The transient experiments following slow infusion of 10 mg/kg MKT-077 are not yet complete, but it similarly has shown no significant change in blood pressure, tumor PO_2, or blood flow (data not shown). We are in the process of completing this dose group.

To address Objective 2, we used oxygen microelectrodes to measure PO_2 histograms in the tumors either 2 or 24 hours after infusion of MKT-077. From the histograms we determined the mean PO_2, the median PO_2, and the hypoxic fraction (% PO_2 values below a certain PO_2 value). We performed three experiments at 24 hours post-infusion, but they showed no obvious shift in the

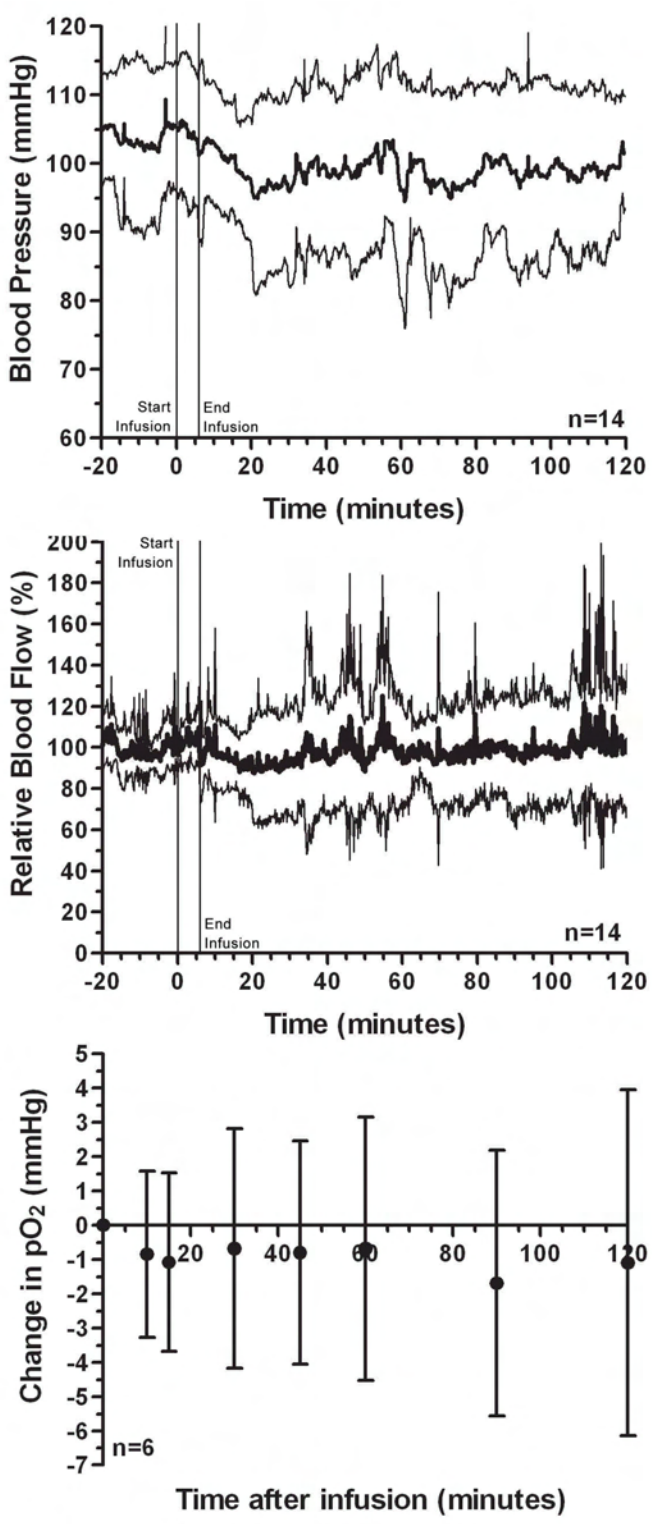

Figure 8. The effects of IV infusion of 7.5 mg/kg MKT-077 at a rate of 1.25 mg/(kg min) on blood pressure (top), blood flow (center), and pO_2 change (bottom). Mean ± SD, n given in panel.

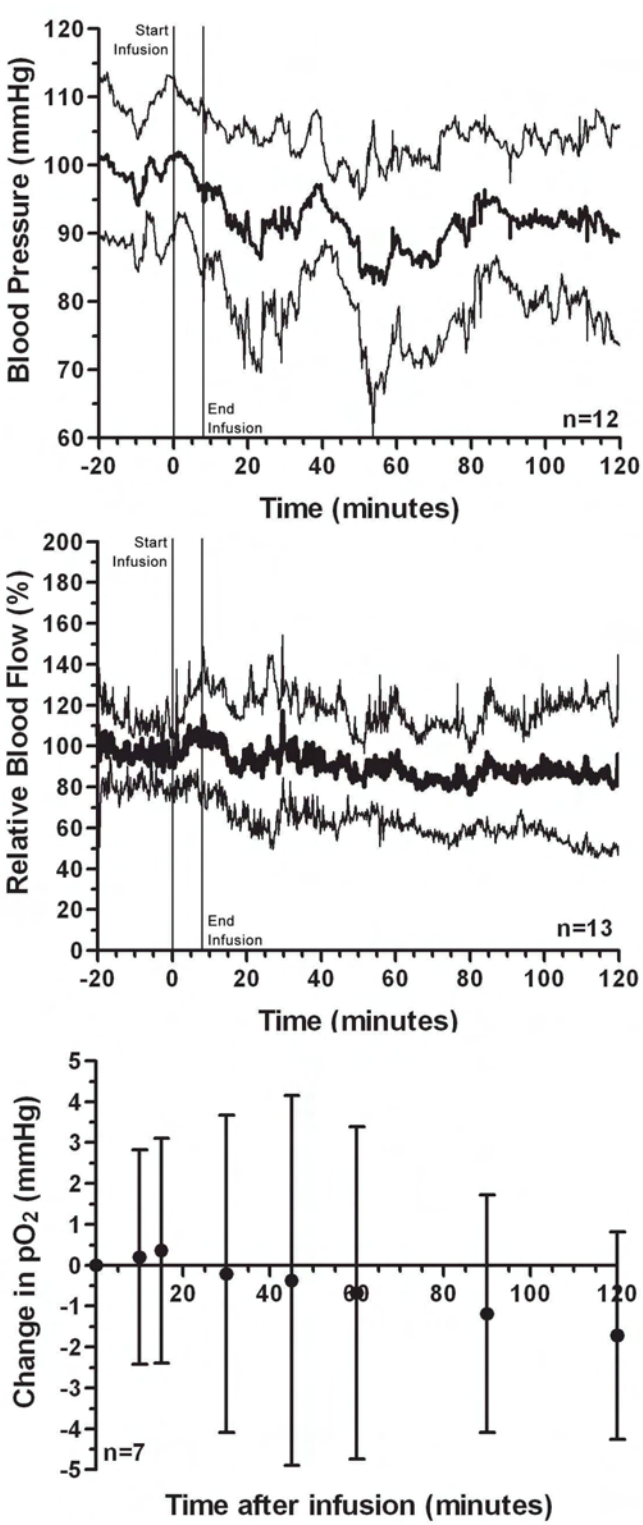

Figure 9. The effects of IV infusion of 10 mg/kg MKT-077 at a rate of 1.25 mg/(kg min) on blood pressure (top), blood flow (center), and pO_2 change (bottom). Mean ± SD, n given in panel.

distributions. Given all of our transient data immediately following the infusion, we decided to measure histograms in rats immediately following the infusion for at least two hours. This also allowed us to benefit from the statistical power of paired experiments, since histograms before and after MKT-077 infusion could be measured in the same rat and could be compared in a paired fashion. The results of these experiments are presented in Figures 11, 12, 13, and 14. Panels A and B show the overall histograms before and after MKT-077 infusion, respectively. These histograms include every point measured in all the tumors in the group. Panels C and D in each figure show the pre- and post-infusion mean and median PO_2 values for each tumor in the group, respectively. Each rat/tumor is a different color, and the pre- and post-values for each rat are connected by a line. As expected, infusion of saline had no significant effect on the tumor PO_2 distribution (data not shown). Fast infusion [1.25 mg/(kg min)] of 7.5 mg/kg or 10 mg/kg MKT-077 failed to significantly increase tumor PO_2 , i.e., there was no right shift in the histograms and no difference between pre-infusion and post-infusion descriptive parameters (Figures 11 and 12). In both cases, mean and median tumor PO_2 in individual rats decreased, increased, or remained constant following the infusion. Similarly, slow infusion [0.125 mg/(kg min)] of 7.5 mg/kg MKT-077 resulted in no significant changes in the tumor PO_2 distribution (Figure 13). However, slow infusion of 10 mg/kg MKT-077 had an impressive effect on the PO_2 histogram (Figure 14). The overall histograms, which include every point measured in the six tumors, clearly shifted to the right following the MKT-077 infusion (Figures 14A and 14B). The median PO_2 of these overall histograms increased from 0.7 mm Hg to 2.6 mm Hg. When paired statistical comparisons were performed on the individual parameters, statistically significant improvements in tumor oxygenation were found (Figures 14 and 15). The mean PO_2 increased significantly in all 6 tumors (Figure 14C, p=0.026). The median PO_2 increased in all six tumors as well, although this increase was not statistically significant (Figure 14D, p=0.107). We believe this may have been due to the fact that these tumors were extremely hypoxic. Five of the six tumors had median PO_2 values below 1 mm Hg (Figure 14D). It is very difficult to increase such low PO_2 values. Nevertheless, there were significant decreases in the hypoxic fraction, which is a key parameter in the

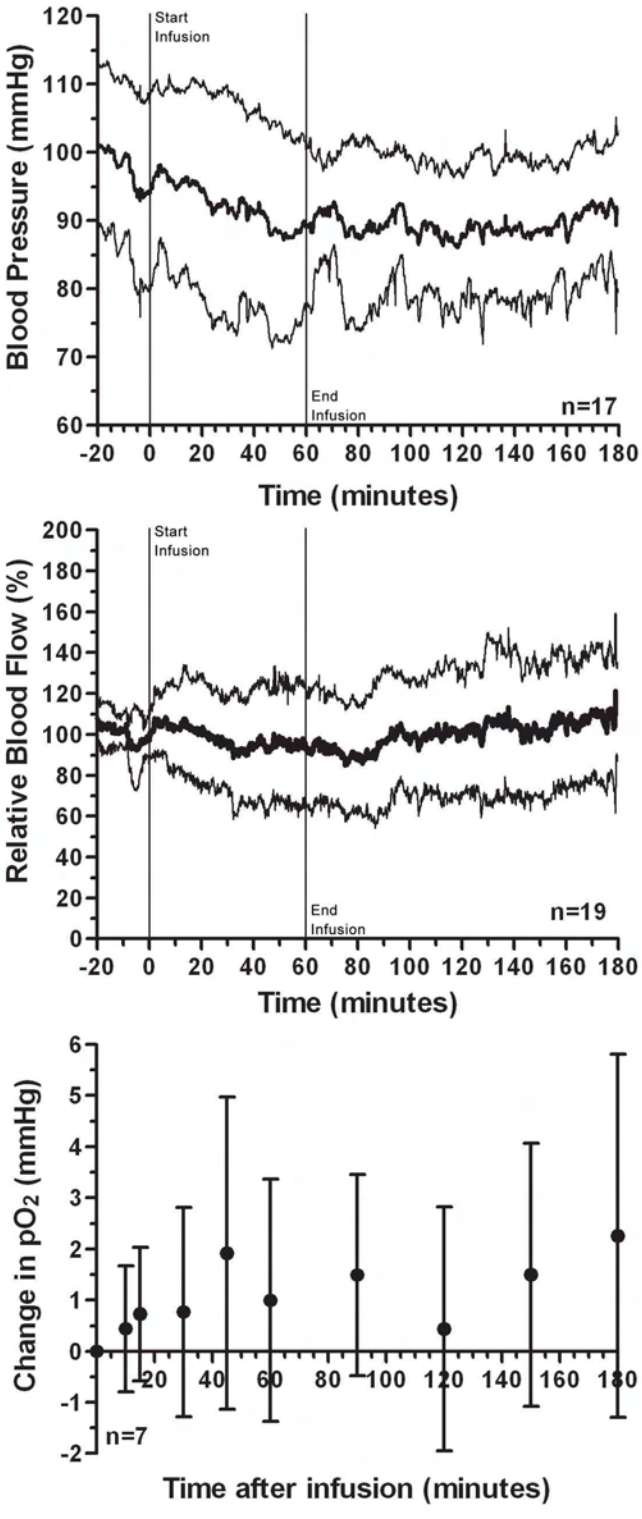

Figure 10. The effects of IV infusion of 7.5 mg/kg MKT-077 at a rate 0.125 mg/(kg min) on blood pressure (top), blood flow (center), and pO_2 change (bottom). Mean ± SD, n given in panel.

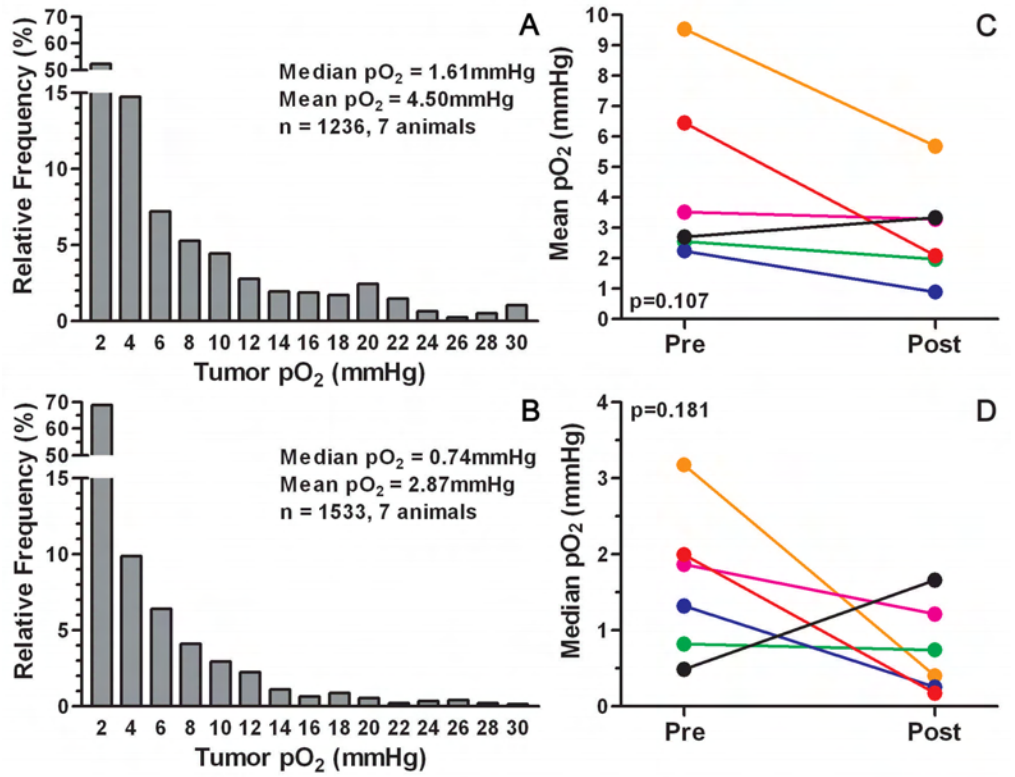

Figure 11. The effect of fast 7.5mg/kg MKT-077 infusion [1.25 mg/(kg min)] on tumor pO_2 distribution. A: pre-infusion pO_2 histogram, B: post-infusion pO_2 histogram, C: Pre- and post-infusion mean pO_2, D: Pre- and post-infusion median values. p values calculated by the Student's paired t-test. N = 6 rats.

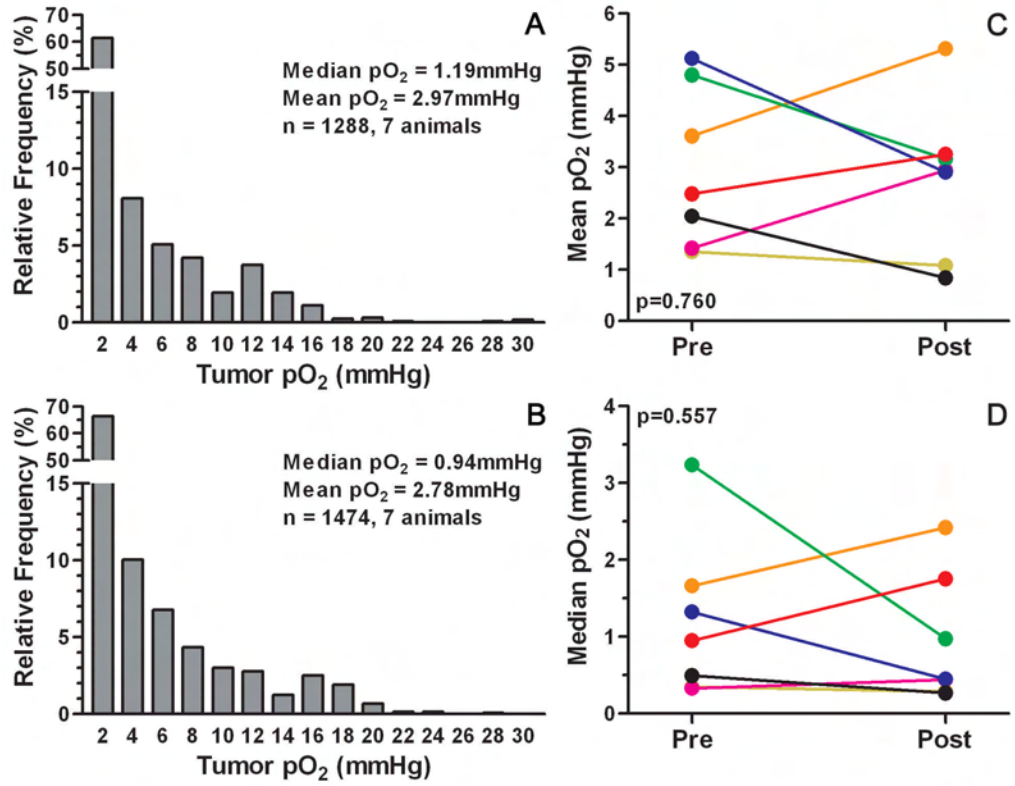

Figure 12. The effect of fast 10 mg/kg MKT-077 infusion [1.25mg/kg/min] on tumor pO_2 distribution. A: pre-infusion pO_2 histogram, B: post-infusion pO_2 histogram, C: Pre- and post-infusion mean pO_2, D: Pre- and post-infusion median values. p values calculated by the Student's paired t-test. N = 6 rats.

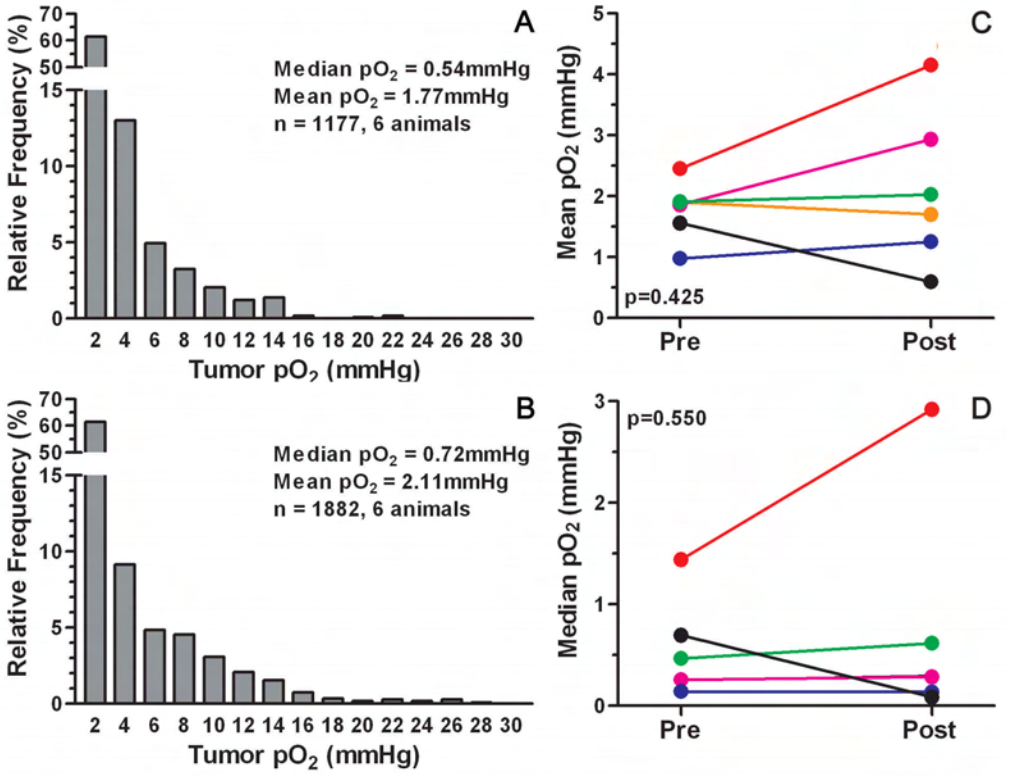

Figure 13. The effect of slow 7.5 mg/kg MKT-077 infusion [0.125mg/kg/min] on tumor pO_2 distribution. A: pre-infusion pO_2 histogram, B: post-infusion pO_2 histogram, C: Pre- and post-infusion mean pO_2, D: Pre- and post-infusion median values. p values calculated by the Student's paired t-test. N = 6 rats.

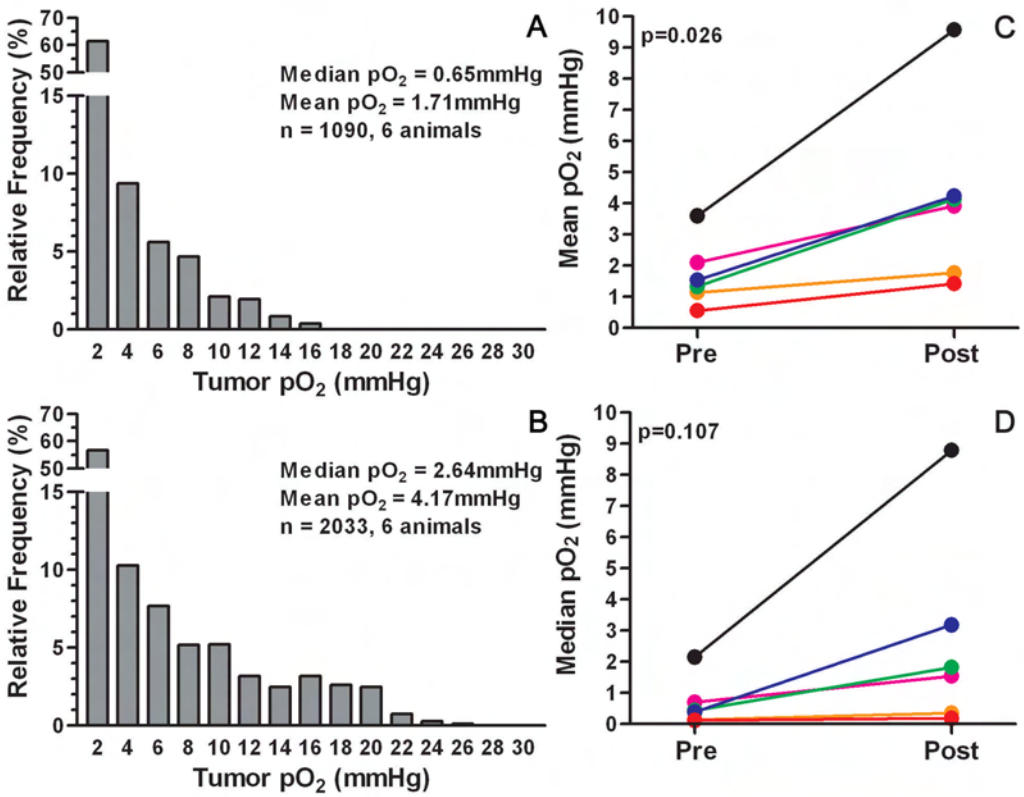

Figure 14. The effect of slow10 mg/kg MKT-077 infusion [0.125mg/kg/min] on tumor pO_2 distribution. A: pre-infusion pO_2 histogram, B: post-infusion pO_2 histogram, C: Pre- and post-infusion mean pO_2, D: Pre- and post-infusion median values. p values calculated by the Student's paired t-test. N = 6 rats.

tumor response to radiation (Figure 15). Slow infusion of 10 mg/kg MKT-077 significantly decreased the percentage of values less than 2.5 and 5 mm Hg, while the decrease in percentage of values less than 10 mm Hg was not quite significant (p=0.081). This means that this dosing schedule of MKT-077 was able to shift very low PO_2 values significantly to the right into the range of improved radiosensitivity, from <2 mm Hg to >5 mm Hg. We believe the results would be even more impressive if the tumors had not been so extremely hypoxic. For example, in the one relatively well-oxygenated tumor (black points in Figures 14 and 15), the median PO_2 increased from 3.5 mm Hg to over 9 mm Hg (Figure 14), while the hypoxic fraction (% < 5 mm Hg) dropped from 70% to 40%. These results show that MKT-077 can have significant *in vivo* effects on tumor PO_2, and that the dosing schedule is extremely important.

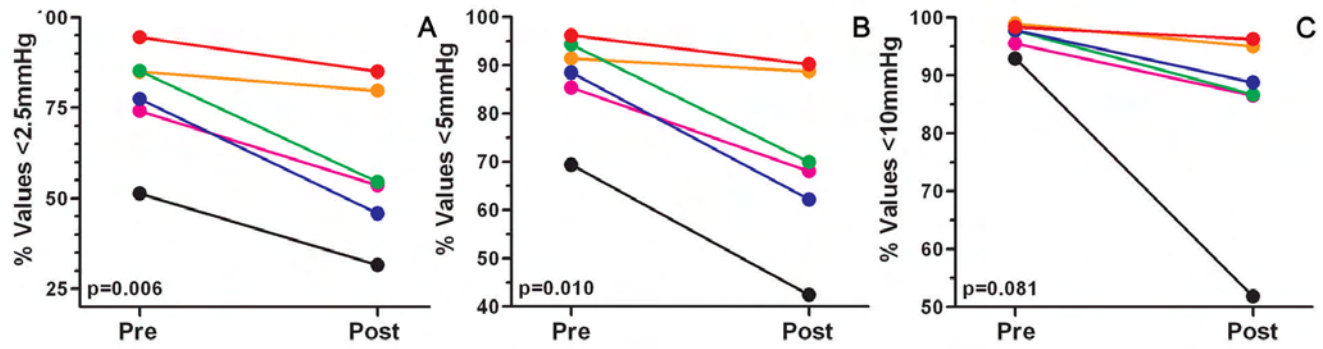

Figure 15. The effect of slow 10 mg/kg MKT-077 infusion [0.125mg/kg/min] on tumor pO_2 hypoxic fraction defined as the percentage of values less than 2.5 mm Hg (A), 5 mm Hg (B), and 10 mm Hg (C). Data is normalized to account for sampling differences. Plots are color matched for each animal. p values determined using the Student paired t-test.

In order to verify that tumor uptake of MKT-077 was responsible for the observed effects on tumor PO_2, we harvested the tumors, extracted the MKT-077, and determined the tumor concentration of MKT-077 spectrophotometrically. On average the fast infusion schedules and the slow infusion of 7.5 mg/kg MKT-077 resulted in tumor concentrations between 1 and 1.5 ng MKT-077/mg tumor (Figure 16). These values were not statistically different from one another. Slow infusion of 10 mg/kg, however, resulted in a significantly higher tumor level of MKT-077 of 2.2 ng MKT-077/mg tumor on average. In

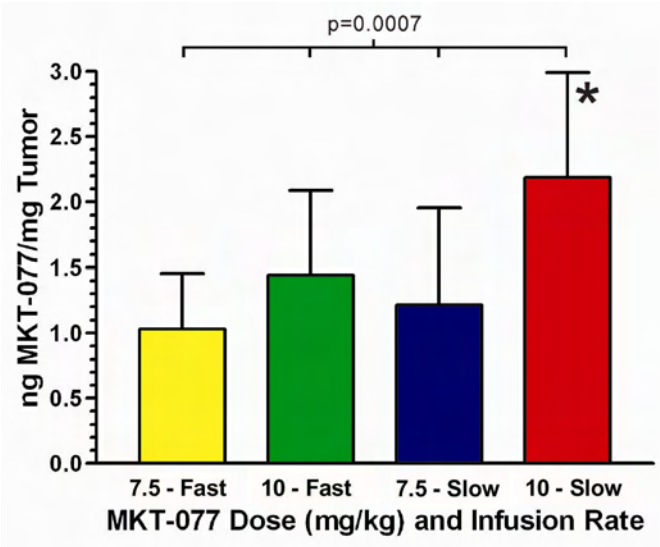

Figure 16. The effects of infusion rate and MKT-077 dose on tumor uptake amounts. Infusing the high dose of MKT-077 at the slow infusion rate [10 mg/kg at 0.125mg/(kg min)] yields the greatest uptake over all other groups. p value calculated using an ANOVA with Bonferroni's multiple comparison test.

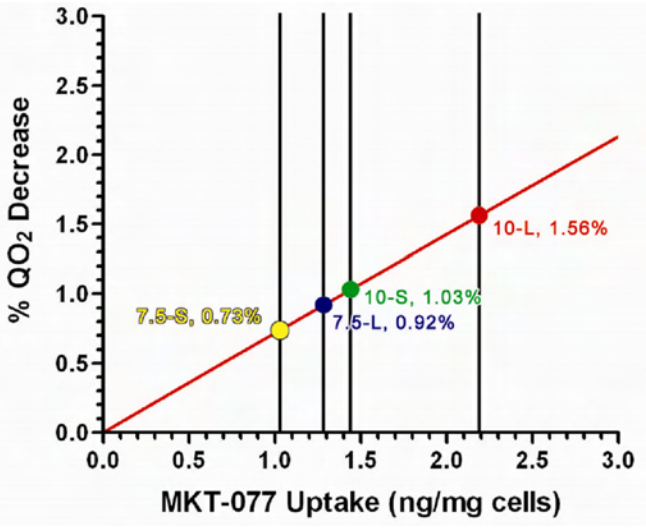

Figure 17. The proposed change in in vivo oxygen consumption by MKT-077 uptake amount. The correlation line adapted from in vitro uptake and metabolic inhibition studies (Figure 7A). Abscissa was converted from ng MKT-077/100,000 cells to ng of MKT-077/mg cells to account for tumor sample weights.

order to estimate the decrease in oxygen consumption that would result from this level of MKT-077 accumulation, we used the line generated from the *in vitro* relationship between consumption decrease and drug uptake by R3230Ac cells raised in 1% O_2 (Figure 7A). To make the comparison, the abscissa was converted from ng MKT-077/100,000 cells to ng of MKT-077/mg cells to account for tumor sample weights. The results indicate that the levels of MKT-077 in the tumors *in vivo* would yield consumption decreases of only around 0.6-1.5% (Figure 17). Nevertheless, the 1.5% decrease in consumption resulting from the slow infusion of 10 mg/kg MKT-077 was large enough to improve tumor oxygenation. Clearly the window of opportunity with MKT-077 is still relatively large. The challenge will be to increase delivery of the drug to the tumor while minimizing any systemic effects, e.g., blood pressure decreases.

In summary, the results of Task 3 demonstrate that MKT-077 can function as a metabolic inhibitor *in vivo* and is capable of significantly increasing tumor PO_2, even at low concentrations in severely hypoxic tumors. The results validate one of our prime hypotheses that MKT-077 can increase tumor oxygen levels. We also showed that infusion of MKT-077 using our four dosing schedules did not alter tumor blood flow and had minimal effects on blood pressure. The lack of an effect of MKT-077 on tumor blood flow is significant, because any significant decrease in perfusion would decrease oxygen supply and counteract the beneficial effect of the inhibition of oxygen metabolism. These results are extremely promising, especially since we estimate that we only delivered small amounts of the drug using our dosing schedules. If we can improve delivery and increase tumor levels of MKT-077, we believe that we can improve tumor oxygenation even more in these severely hypoxic tumors. The data also suggest that the drug might be even more effective in tumors that are less severely hypoxic. Most of the R3230Ac tumors in this study had median PO_2 values below 2 mm Hg. It would be interesting to explore this drug in other tumors that are less hypoxic. Given these positive results, we have requested a no-cost extension to complete Task 3 and investigate another dosing schedule in the upcoming year.

Revised Task 4. To determine if MKT-077 infusion before single-dose radiation therapy can delay the growth of orthotopic rat breast cancer tumors better than either radiation or drug treatment alone (Months 43-48).

Model: We will grow R3230Ac rat mammary carcinomas orthotopically in the mammary fat pads of Fischer 344 rats. We estimate requiring 45 rats for this study.

Methods: We will irradiate established R3230Ac tumors that have either received MKT-077 or the saline vehicle. Parallel groups will be sham irradiated. Tumor volume will be measured 3 times per week until the tumors have reached 5 times the initial volume or for a maximum of 60 days.

Objective:

1) Determine if MKT-077 infusion before irradiation will result in significant tumor growth delay compared to the other three groups: saline with sham irradiation, MKT-077 with sham irradiation, and saline with irradiation.

Progress on Task 4: In the original Statement of Work, these experiments were not scheduled to be performed until the end of Year 3 of the grant. Because we only recently found an effective dosing schedule, we have not yet performed these experiments. Since we have demonstrated *in vivo* efficacy of the drug as a metabolic inhibitor, but have not yet optimized the dosing schedule, we have requested a no-cost extension to explore another dosing schedule. If we can find a dosing schedule that is effective at decreasing the hypoxic fraction in the R3230Ac tumors even more, we are also proposing to use some of the extended funds to perform the tumor growth delay study in Task 4.

KEY RESEARCH ACCOMPLISHMENTS

- Last year we completed the *in vitro* studies investigating the uptake of MKT-077 by R3230Ac cells *in vitro*. We showed that MKT-077 is rapidly taken up by the R3230Ac rat breast cancer cells, in a dose-dependent fashion. We have now demonstrated that cells raised under hypoxic conditions take up less MKT-077 than cells raised on air. In addition, low oxygen levels inhibit the uptake of MKT-077, regardless of the original growth conditions. These findings may have important implications for *in vivo* use of the drug.
- Last year we also completed the *in vitro* studies investigating the effect of MKT-077 on oxygen metabolism in R3230Ac cells. We have shown that MKT-077 effectively inhibits oxygen consumption in the R3230Ac cells in a dose-dependent manner, up to a maximum of ~70% inhibition at 6 μg/ml. The relative inhibition of oxygen consumption, but not the absolute inhibition, is independent of the oxygen level at which the cells were grown. Cells grown on air show a greater absolute inhibition of consumption rate than cells grown under hypoxic conditions.
- As a related point, we showed that cells grown on air have a higher basal oxygen consumption rate than cells grown at either 1% or 2.5% O_2, even though there is no difference in mitochondrial protein content among the cells. This is an interesting finding that could be pursued further.
- We showed a direct correlation between cellular MKT-077 uptake and the magnitude of inhibition of cellular oxygen consumption.
- **Most importantly**, we have shown that slow intravenous infusion of 10 mg/kg MKT-077 over 60-80 minutes significantly increases PO_2 in R3230Ac mammary adenocarcinomas growing orthotopically in Fischer 344 rats. We correlated the change in PO_2 to increased tumor MKT-077 concentration, compared to those achieved by other infusion schedules. This is proof that MKT-077 can increase tumor PO_2 *in vivo* and might be a useful radiosensitizer.

REPORTABLE OUTCOMES

Due to the fact that we only recently completed the *in vitro* oxygen consumption studies in the hypoxic cells, we have not yet published any results on the cellular uptake of MKT-077 and tresultant inhibition of cellular respiration. The graduate student, John Chunta, who performed most of the experiments is currently working on the manuscript , and we plan to publish it in the near future. We also expect to publish the *in vivo* work within the next six months. We need to complete the transient slow 10 mg/kg MKT-077 infusion experiments.

CONCLUSIONS

In this third year of the grant, we have had significant success. We have further characterized the uptake of MKT-077 by the rat breast cancer cell line, R3230Ac. Importantly, we have shown that the uptake of this drug is oxygen dependent. The uptake not only depends on the oxygen level at which the cells were grown, it also depends on the oxygen level at the time of drug exposure. This could have significant impact on the uptake of this drug *in vivo*. We have also completed our investigation of MKT-077-induced inhibition of oxygen consumption in the R3230Ac cell line *in vitro*. The drug is capable of inhibiting oxygen consumption in this cell line in a dose-dependent manner, whether the cells are raised under normoxic or hypoxic conditions. There is a direct link between the amount of MKT-077 taken up by the cells and the magnitude of the oxygen consumption inhibition. Finally, and most importantly, we have also demonstrated that the drug can significantly improve tumor PO_2 *in vivo* when slowly infused at a dose

of 10 mg/kg over about an hour. We found that the dosing schedule is very important in achieving effective levels of MKT-077 in the tumor. We believe that we could further optimize this effect by increasing the dose and slightly slowing the infusion rate. We have requested a no-cost extension of this grant to further pursue this objective, as well as potentially completing the radiation study proposed in Task 4.

The ability of MKT-077 to inhibit oxygen consumption *in vitro* is very impressive. Two μg/ml of MKT-077 was able to decrease oxygen consumption in R3230Ac cells by about 40% after one to two hours of exposure. Higher concentrations inhibited the consumption much more quickly and to a greater extent. Secomb and coworkers demonstrated theoretically that inhibition of consumption by 30% was sufficient to eradicate hypoxia in a model tumor.[6, 7] Thus, even relatively low doses of MKT-077 are able to inhibit R3230Ac metabolism to an extent that is physiologically relevant. This conclusion was verified by our *in vivo* study, in which we were able to achieve maximal tumor drug concentrations of about 2 ng/mg tumor. Based on our *in vitro* data this probably inhibited consumption on the order of 1.5%. Nevertheless, this inhibition was significant enough to improve tumor PO_2.

Our *in vivo* results are the most important of the past year. We showed that infusion of 10 mg/kg MKT-077 over 60 minutes can improve *in vivo* tumor PO_2 shortly after infusion, which is consistent with the rapid inhibition of tumor cell oxygen consumption demonstrated *in vitro*. The fact that we were operating at the edge of the efficacy range for MKT-077 is also noteworthy. The other three infusion schedules resulted in tumor drug concentrations between 1 and 1.5 ng/mg tumor, and showed no consisitent effect on tumor PO_2. The slow infusion of 10 mg/kg MKT-077 resulted in tumor levels of 2.2 ng/mg tumor, and this small increase in drug delivery was enough to significantly decrease tumor hypoxic fraction. This suggests that small improvements in drug delivery could have important implications for improving tumor oxygenation further. If we can increase tumor PO_2 a little more, it would be useful to attempt the radiation study in Task 4. We have requested a no-cost extension to complete Task 3 and perhaps perform the radiation study.

Overall, I believe this project has been a success. We have proven the first three hypotheses as outlined in the Statement of Work and have demonstrated efficacy of MKT-077 as a metabolic inhibitor *in vivo*. We hope to show that the MKT-077-induced changes in PO_2 are enough to impact radiation response. We believe this drug should be investigated further as a potential radiosensitizer in breast cancer and other tumors as well.

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APPENDICES

None.